

MEASUREMENT OF MUTATION LOAD USING THE p53 GENE
IN HUMAN CELLS FROM PARAFFIN EMBEDDED TISSUES

[0001] This application claims priority from U.S. Provisional Application No. 60/246,582, filed November 8, 2000.

BACKGROUND OF THE INVENTION

[0002] This invention relates to a method for determining the extent and nature of mutations of somatic origin. The method is capable of utilizing as little DNA as that obtained from a single cell, and may reveal predisposition to elevated spontaneous mutations as well as prior carcinogen exposure. Thus the invention provides a useful tool for determining cancer risk or monitoring the effectiveness of cancer therapy.

[0003] Somatic mutations can compromise genome integrity. Mutation analysis of DNA from a single somatic cell or a small group of cells is a useful procedure, having a number of applications. For example, analysis of somatic mutations within single cells permits examination of "mutation load," defined herein as the overall mutation frequency and alterations in mutation pattern and spectrum. These measurements in normal tissues can identify individuals with a high mutation load who are at an increased risk for cancer. Since mutation patterns can vary dramatically with the type of mutagenic insult or defective repair system, they can provide clues to identify mutation sources and/or mechanisms. In addition, mutation analysis of a single cell or small group of cells may be useful for identifying specific mutations that may be associated with a potentially pathological event or a general tool in molecular pathology.

[0004] It has become clear over the last decade that cancer is, in essence, a genetic disease resulting from accumulation of mutations in specific oncogenes and tumor suppressor genes, either through inherited or somatic origins. The p53 tumor suppressor gene is a critical regulator of tumorigenesis and is mutated in 50% of human cancers. Levine, A.J., "The p53 tumor-suppressor gene", N. Engl. J. Med. 326:1350-1352 (1992); Hollstein, M., Sidransky, D., Vogelstein, B., Harris, C.C., "p53 mutations in human cancer", Science 253:49-53 (1992). Moreover, individuals with Li-Fraumeni syndrome, who inherit a p53 mutant allele, develop multiple cancers at an early age. Malkin, D., et. al., "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms", Science 250:1233-1238 (1990). Additionally, transgenic mice with a nullizygous or a mutant p53 gene develop normally but develop cancers within 3 to 6 months after birth. Donehower, L.A., et. al., "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours", Nature 356:215-221 (1992). Furthermore, the p53 gene is associated with responses to various cancer treatments, including, but not limited to, radiation, cytotoxic drug treatment and gene therapy treatment. Lowe, S.W., et. al., "p53 status and the efficacy of cancer therapy *in vivo*", Science 266:807-810 (1994).

[0005] The p53 gene is located in a 20kb region of chromosome 17p13.1. The gene contains 11 exons which encode a nuclear phosphoprotein of 393 amino acids that is expressed in all cell types. The p53 protein can be divided into three major regions based on function. Prives, C., "How loops, beta sheets, and alpha helices help us to understand p53", Cell 78:543-546 (1994). The acidic amino-terminal contains the transcriptional activation region and a binding site for the product of the mdm2 gene. The central region

PCNA promoter by p53", Biochem. Biophys. Res. Commun. 203:133-140 (1994).

However, tissues occasionally contain normal cells that exhibit accumulated p53 protein. Some of these cells stabilize wild type (wt) protein while others may contain mutant protein. Furthermore, a mutation in one of the two alleles may not always lead to p53 protein accumulation as it is occasionally seen in tissues of Li-Fraumeni patients. As a result, the p53 positive stained cell does not necessarily contain a mutation in the p53 gene. The p53 gene is also activated by hypoxia, heat shock, exposure to nitric oxide and other stresses.

[0008] To identify individuals who are predisposed to elevated spontaneous mutations or who have had previous carcinogen exposure, it is advantageous to use the smallest amount of PCR template that could result in an accurate picture of mutation load. HotStart PCR, which helps to prevent the formation of primer dimer, permits amplification of more dilute template. Using this technique, a 140 bp PCR segment was amplified from an extracted single DNA template using 60 cycles. Vogelstein, B., Kinzler, K.W., "Digital PCR", Proc. Natl. Acad. Sci. U.S.A. 96:9236-9241 (1999). This technique involves the fewest number of PCR cycles required for dependable results reported to date.

[0009] Thus, there exists a need for a method to assess mutation load, both in subjects which do not yet exhibit signs of the disease, and subjects which are presently being treated using known cancer therapy to assess efficacy of treatment.

SUMMARY OF THE INVENTION

[00010] In accordance with the present invention it has been discovered that the p53 gene is a useful reporter of somatic cell mutation analysis, including determination of mutation load. In one embodiment, the invention is a method for determining mutation load which involves identifying a somatic cell that contains accumulated levels of p53, amplifying DNA from the p53 gene in such cell and determining the frequency or nature of mutations in the amplified DNA. In another embodiment, the p53 gene is obtained from a single somatic cell.

[0011] In a further embodiment, the method of this invention involves a method for determining mutation load by identifying a somatic cell that contains accumulated levels of both p53 and altered levels of a protein which is the product of a gene whose expression is regulated by p53, amplifying DNA of the p53 gene in such cell, and determining the frequency or nature of mutations in the amplified DNA. Cells containing accumulated levels of p53 and altered levels of such other protein advantageously may be identified by immunohistochemical staining using antibodies to those proteins. Mutations may be identified by sequence analysis of the amplification product.

[0012] As described in greater detail below, the combination on microdissection of single cells with well preserved and intact genomic DNA and efficient amplification makes possible a mutation load assay from ethanol-fixed paraffin-embedded tissues. A robust method involves reproducible double immunohistochemical staining, efficient microdissection of single cells under conditions that preserve genomic DNA integrity, and efficient amplification of the p53 gene with low false negatives and low false positives. The method of this invention has been used successfully and analyze DNA templates as

large as 1 kb, preferably as large as 2 kb. The method has been used to identify missense mutations in single cells from normal colon and other tissues. Specifically, mutations were identified in 50% of the amplified samples by sequence analysis of exons 5 to 9 of the p53 gene. In addition, rates of allele dropout and polymerase error, important to estimates of mutation load, were examined.

[0013] This invention is useful for assessing cancer risk and prognosis and monitoring the effectiveness of cancer therapy. The method also can be used to monitor the mutational status of individuals over extended periods of time, such as individuals who have environmental or inherited cancer risks. Changes in or accelerated progression of mutation load can signal increased risks for cancers or other cellular abnormalities.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1, Panel A shows normal colon tissue with crypt cells stained positive for PCNA and for p53 (black arrow). Figure 1, panel B shows normal lung tissue with bronchial epithelial cells. Cells are stained for PCNA (white arrow), and p53 (black arrow). Figure 1, panel C shows benign proliferative lesion of the mammary gland. Cells are stained for p53 (white arrow) and for p53/PCNA (black arrow).

[0015] Figure 2 shows single cell amplification by Stimulated-PCR, the technique discussed in Example II below. A 2 kb region of the p53 gene was amplified with 0.6 μ l of p53(12983)30D (SEQ. ID. NO. 1) and p53(15036)33U (SEQ. ID. NO. 3) from genomic DNA of single microdissected cells for 40 cycles. Lanes 1 to 12 are single cell amplifications. Eleven out of 12 single cells were amplified. Lane 13 = no DNA, Lane 14 = positive control with 20 cells, M = DNA marker.

[0016] Figure 3 shows examples of identified somatic mutations. Panels A through D are examples of identified somatic mutations in sequence analysis. Mutations of C-T, G-A, T-A and T-C were identified with second peak heights from 100% to 30% of the wild type. Causative mutations were found in 50%-70% of the amplified single cells. All mutations were confirmed by an additional sequencing in the opposite direction.

DETAILED DESCRIPTION OF THE INVENTION

[0017] Somatic tissue for examination by the method of this invention may be obtained from any tissue source, e.g., colon, prostate, breast, skin, lung, etc. The tissue is prepared for identification of cells that have accumulated p53, as such cells are likely to have mutations in the p53 gene and other genes. Any method of preparing tissue samples which permits microdissection of cells and minimizes damage to nuclei may be used. A preferred method for tissue preparation involves ethanol fixation, paraffin embedment and EDTA steam heating to unmask antigenic sites. The method is described below in connection with a preferred embodiment.

[0018] Ethanol is a preferred tissue fixative, because it precipitates antigens and does not cause DNA crosslinking, as does formalin. Following ethanol fixation, tissue is embedded in paraffin and sliced in thin sections by standard procedures. Routine pathology sections conventionally are between 4 μ m and 5 μ m in diameter. When using this diameter, tissue sections consist not only of undamaged sections, but also contain some damaged nuclei, resulting in allele dropout. To enhance the possibility of obtaining undamaged nuclei and reducing the risk of dissecting damaged cell nuclei, large sections,

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e.g., about 6 μm or greater are preferred. Steam heating using an EDTA buffer has been found to yield reliable immunohistochemical staining and intact DNA. Taylor, C.R., Shi, S.R., Cote, R.J., "Antigen retrieval for immunohistochemistry status and need for greater standardization", Applied Immunohistochemistry 4:144-146 (1996). Suitable conditions include a buffer containing 1mM EDTA (pH 8.0) at 96 to 100° C and heating for 5 minutes. Alternatively, steam heating can be performed using 20 mM HEPES/1mM EDTA buffer (pH 8.1) with a pK much less affected by high temperature. Using these process enhancements, the size of the single stranded DNA has been determined to have an average length of 20 kb.

[0019] Cells that accumulate p53 or have altered levels of a protein which is the product of a gene whose expression is regulated by p53 may be identified by immunohistochemical staining. Monoclonal antibodies that may be used for this purpose are available commercially. See Examples, infra. Because mutant p53 accumulates in cells, staining for this protein is useful for identifying cells in which mutations have occurred. The p53 protein regulates the expression of a number of other genes, including PCNA, mdm2 and vEGF. Thus the levels of the proteins that are the products of these genes often are altered in cells containing mutant p53. For example, p53 down regulates PCNA, and mutant p53 may result in accumulated levels of PCNA in cells. The levels of other proteins under p53 control may be increased or decreased, depending on the mechanism of the control. The use of the levels of expression of one or more of such secondary proteins assists in differentiation between cells having enhanced levels of wt p53 resulting from natural physiological induction and the cells of interest having accumulated levels of mutant p53.

[0020] After preparation and staining of the paraffin-embedded tissue sections, single cells that stain positive for p53 and/or PCNA are microdissected from nontumorous tissues. Microdissection is performed by standard procedures.

~~[0021] Mutation analysis, including determination of mutation load, advantageously is determined by amplification and analysis of DNA from a single cell. The DNA is amplified by any procedure that efficiently reproduces DNA from the low template concentrations obtained from a single cell. A preferred amplification procedure, referred to herein as "Stimulated PCR," has been found to yield sufficient DNA for sequence analysis using as few as 40 cycles of amplification. This PCR process differs from known processes in that it substantially reduces the threshold effect of the template concentration on PCR efficiency. Additional advantages of Stimulated-PCR may include: inhibition of adsorbance of the template to the tube surface; protection against minimal DNAase activity; addition of false priming sites for spurious extensions; activation by binding DNA polymerase; or direct stimulation of extension by DNA polymerase. Stimulated PCR is described in detail in the Examples, infra. In general, the technique is characterized by the use of a combination of a Taq polymerase High Fidelity and Taq DNA polymerase and by the incorporation of mouse genomic DNA having an average size of more than about 20 kb. The addition of mouse genomic DNA allows a wider range of annealing temperatures, a wider range of primer concentrations, less primer dimer formation, and higher product yield. Similar effects were observed by adding or supplementing bovine serum albumin (BSA), probably because BSA protein assists in keeping DNA polymerases in active forms.~~

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[0022] Changes in the use of High Fidelity enzymes, commercially available and utilized by those of skill in the art, improve amplification yields. The highest yields are found when the High Fidelity enzymes are used in higher amounts than those typically used, e.g., at about 4-fold the amount recommended by the manufacturer (2.5 U Taq/GB-D DNA polymerases per 25 μ l of reaction). Additionally, mixing 1 U of Platinum Taq with 1 U of the High Fidelity enzymes, which increases the unit ratio of Taq to GB-D by 2 fold, behaves better than the High Fidelity enzymes alone, indicating that not only the total units, but also the relative ratios of the enzymes are important. Another improvement that may be used to increase the fidelity of the amplification and to minimize primer-dimer formation is the incorporation of a Taq antibody to inactivate Taq DNA polymerase at room temperature. This improvement is used in so-called HotStart PCR. In the present method, it has been effective in preventing primer dimer formation using 40 to 45 cycles.

[0023] Primer design is an important factor in Stimulated PCR. Analysis of any region of the p53 gene may be used in the method of this invention. In a preferred embodiment, sequences from exons 5 to 9 of the p53 are amplified and analyzed, as a large majority of mutations occur in these regions. Table 1 presents a list of primers which have been utilized during development of this invention. The following primers have been found particularly effective for Stimulated-PCR:

GCCGTCTTCCAGTTGCTTTATCTGTTCCTACT (SEQ. ID. NO. 1);

CCTGATGGCAAATGCCCAATTGCAGGTAA (SEQ. ID. NO. 2); and

GTCAAGTAGCATCTGTATCAGGCAAAGTCATAG (SEQ. ID. NO. 3). Primer

TGTTCACTTGTGCCCTGACTTTCAACTCTG (SEQ. ID. NO. 4) was used in

conjunction with CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2) for half-nested PCR. The specific physical properties of these sequences are set forth in Table 1. Primer design was achieved using a strict criteria to ensure PCR specificity and efficiency, which is explained further in Example II below. For instance, it was discovered that primers with AA dinucleotides at the 3' end formed fewer dimers.

Table 1. List of primers in the *p53* gene

p53(13950)20D	AGGTCTCCCCAAGGC GCACT [SEQ. ID NO. 8]	20	59.5	65	D sequencing
p53(14155)20U	GGGGCACAGCAGGCC AGTGT [SEQ. ID NO. 9]	20	61.7	70	U sequencing
p53(14506)19D	GGAGAGACCGGCGC ACAGA [SEQ. ID NO. 10]	19	58.4	68.4	D sequencing
p53(14487)24U	CGGCATTTTGAGTGT TAGACTGGA [SEQ. ID NO. 11]	24	57.1	45.8	U sequencing

[0024] a. The sequence of the *p53* gene was from a revised version of X54156 in GenBank. As an example for *p53* (12983)30D, *p53*= the *p53* gene, (12983)30D=5' end of the primer begins at 12983 base position, and the length is 30 bases "downstream" (D) (i.e., in the direction of transcription). The precise sizes and locations of the PCR fragment can be obtained from the informative names.

[0025] b. T_m of the primer was estimated by the nearest neighbor method at 50 mM KCl and 250 pM DNA and T_m of the PCR segment was estimated by the formula of Wetmur: $T_m^{product} = 81.5 + 16.6 \log[K^+ = 0.05 \text{ M}] + 0.41 (\%G + \%C) = 675/\text{length}$.

[0026] c. primer pairs of [SEQ. ID. NO. 1] and [SEQ. ID. NO. 2] and of [SEQ. ID. NO. 1] and [SEQ. ID. NO. 3] were used for Stimulated PCR; primer pair of [SEQ. ID. NO. 4] and [SEQ. ID. NO. 2] were for half-nested PCR, Primers [SEQ. ID. NO. 5] through [SEQ. ID. NO. 11] were for sequencing.

[0027] Because contamination is a significant consideration when only amplifying DNA product from a single cell, various sources of non-specific DNA and excessive PCR amplification must be eliminated. To decrease or eliminate these possible causes of contamination, the following steps were taken: i) negative controls without target DNA template are routinely performed with each assay, and ii) the preparation process was always performed in a special clean room. Moreover, if a number of microdissected

single cells are found to contain different mutations, this is evidence that the finding is not attributable to a common source of contamination.

[0028] Once amplification is completed using the Stimulated-PCR process, the PCR product is sequenced according to techniques known in the art. Sequencing is described in Example III below. Primers that may be used for the sequencing process include:

TGCCCTGACTTTCAACTCTGTCTC (SEQ. ID. NO. 5);

AGGGTCCCCAGGCCTCTGAT (SEQ. ID. NO. 6);

GGCCACTGACAACCACCCTTAA (SEQ. ID. NO. 7);

AGGTCTCCCCAAGGCGCACT (SEQ. ID. NO. 8); GGGGCACAGCAGGCCAGTGT (SEQ. ID. NO. 9); GGAGAGACCGGCGCACAGA (SEQ. ID. NO. 10); and

CGGCATTTTGAGTGTTAGACTGGA (SEQ. ID. NO. 11). Physical properties of these sequencing primers are set forth in Table 1. Table 3 shows the mutations identified using the assay method disclosed herein. Once the mutations are identified, to determine mutation load, an accurate estimate is made of both allele dropout and false positive mutations due to polymerase error. This process is detailed in Example IV.

[0029] Generally, allele dropout may be caused by failure to retrieve the entire cell nucleus due to microdissection, degradation or nicking of the genomic DNA, absorbance of the DNA to the tube surface, preferential amplification of one allele and/or preferential primer extension in the sequencing reaction. To eliminate some of these causes of allele dropout, the paraffin-embedding procedure has been altered, as detailed above, to increase the diameter of the section used. Allele dropout due to nicked template can be kept at a reasonably low level. Table 2 shows the size effect of single-stranded template on allele dropout for amplification of 2kb. The staining process changes discussed above, as well

as addition of mouse genomic DNA or BSA to the digestion reaction, further protect the target DNA from potential degradation and prevent absorbance to the tube surface.

Preferential PCR amplification is diminished by primer design, reaction component changes and optimization of the thermocycling steps.

Table 2. List of Identified Mutations^a

Single stranded template			Average size of single stranded template ^b				
No. nicked strands	No. combinations	No. allele dropout	3 kb	4 kb	6 kb	10 kb	20 kb
			Allele dropout	Allele dropout	Allele dropout	Allele dropout	Allele dropout
0	1	0/1	0%	0%	0%	0%	0.0%
1	4	0/4	0%	0%	0%	0%	0.0%
2	6	2/6	9.9%	12.5%	9.9%	5.1%	1.6%
3	4	4/4	39.5%	25.0%	9.9%	2.6%	0.4%
4 ¹	1	0/1	No ampl	No ampl	No ampl	No ampl	No ampl
		Total	49.8%	37.5%	19.8%	7.7%	2.0%

[0030] a. This estimate only considers the size effect of the single stranded template on allele dropout assuming 100% of amplification efficiency amplification. Allele dropout is defined as that the peak height of one allele is lower than 10% of the second allele at the heterozygote base position.

[0031] b. The average size of single stranded template can be determined in its denatured form on a agarose gel. If the size is 4kb, the 2 kb PCR segment has ½ chance within an integrity template without nicking.

[0032] c. When all the four single stranded templates are nicked, there is only one combination of the four templates to be nicked and no amplification can occur.

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1. **Introduction**
 2. **Method**
 3. **Results**
 4. **Discussion**
 5. **Conclusion**
 6. **References**
 7. **Appendix**
 8. **Table 1**
 9. **Table 2**
 10. **Table 3**
 11. **Table 4**
 12. **Table 5**
 13. **Table 6**
 14. **Table 7**
 15. **Table 8**
 16. **Table 9**
 17. **Table 10**
 18. **Table 11**
 19. **Table 12**
 20. **Table 13**
 21. **Table 14**
 22. **Table 15**
 23. **Table 16**
 24. **Table 17**
 25. **Table 18**
 26. **Table 19**
 27. **Table 20**
 28. **Table 21**
 29. **Table 22**
 30. **Table 23**
 31. **Table 24**
 32. **Table 25**
 33. **Table 26**
 34. **Table 27**
 35. **Table 28**
 36. **Table 29**
 37. **Table 30**
 38. **Table 31**
 39. **Table 32**
 40. **Table 33**
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 42. **Table 35**
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EXAMPLE II

STIMULATED PCR

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segment was estimated by the formula of Wetmur: $T^m_{\text{product}} = 81.5 + 16.6 \log[K^+ = 0.05M] + 0.41(\%G + \%C) - 675/\text{length}$. Wetmur, J.G., "DNA probes: Applications of the Principles of Nucleic Acid Hybridization", Critical Rev. in Biochem and Mol Biol. 26:227-259 (1991), the disclosure of which is incorporated herein by reference. The criteria for specificity included high-specificity with low base-pairing stability at the 3' end, no primer-dimer or hairpin formation more than 3 bases at the 3' end, no homo- or repeat-sequence at the 3' end, and no false priming site more than 7 bases at the 3' end for any strand and any segment. The primer also had no false priming site on the mouse p53 gene to generate spurious products. The primers used are shown in Table 1, above. For Stimulated PCR, primer GCCGTCTTCCAGTTGCTTTATCTGTTCCT (SEQ. ID. NO. 1) was used in conjunction with either CCTGATGGCAAATGCCCCAATTGCAGGTAA (SEQ. ID. NO. 2) and GTCAAGTAGCATCTGTATCAGGCAAAGTCATAG (SEQ. ID. NO. 3). The results of a 2 kb region of the p53 gene amplification with 0.6 μ l of p53(12983)30D (SEQ. ID. NO. 1) and p53(15036)33U (SEQ. ID. NO. 3) from genomic DNA of twelve single microdissected cells for 40 cycles are shown in Figure 2.

[0040] The PCR mixtures contained a total volume of 25 μ l: human genomic DNA from a dissected single cell; #3 Expanded High Fidelity buffer (Boehringer Mannheim); 3.5 mM $MgCl_2$; 500 μ M of each dNTP; 2% DMSO; 0.2 to 0.6 μ M of each of primers; a mixture of 1.25U of Platinum Taq DNA polymerase High Fidelity (Taq/GB-D)/1.25U of Platinum Taq DNA polymerase (GIBCO BRL); 5 μ g of BSA and 25 ng of mouse genomic DNA with the average size more than 20 kb. The cycling conditions included denaturation at 92 °C for 12 seconds, annealing at 60 °C for 20 seconds, and elongation

at 68 °C for 2 minutes for 40 or 45 cycles with a Perkin Elmer GeneAmp PCR system 9700. An additional 20 seconds of denaturation time preceded the first cycle. Two to 4 μ l of the PCR product was electrophoresed through a standard 1% agarose gel and then the gel was stained with ethidium bromide for UV photography by a CCD camera (Bio-Rad Gel Doc 1000) and Multi-Analyst software (version 1.1). Another nested or half nested PCR was performed for 12 to 15 cycles to obtain more product. For this additional half-nested PCR, primers CCTGATGGCAAATGCCCAATTGCAGGTAA (SEQ. ID. NO. 2) and TGTTCACTGTGCCCCTGACTTTCAACTCTG [SEQ. ID NO. 4] were used.

EXAMPLE III SEQUENCE ANALYSIS

[0041] The PCR product was purified for two rounds using Microcon[®] 100 (Amicon) to remove the unincorporated primer and primer dimers. Standard sequence analysis was performed using ABI 377 fluorescence sequencer and BigDye terminator chemistry with AmpliTaq FS DNA polymerase (PE Applied Biosystem). The primers used during the sequencing process were: TGCCCTGACTTTCAACTCTGTCTC (SEQ. ID. NO. 5); AGGGTCCCCAGGCCTCTGAT (SEQ. ID. NO. 6); GGCCACTGACAACCACCCTTAA (SEQ. ID. NO. 7); AGGTCTCCCCAAGGCGCACT (SEQ. ID. NO. 8); GGGGCACAGCAGGCCAGTGT (SEQ. ID. NO. 9); GGAGAGACCGGCGCACAGA (SEQ. ID. NO. 10); and CGGCATTTTGAGTGTTAGACTGGA (SEQ. ID. NO. 11).

[0042] Any second peak was called if its height was more than 10% of the wild type. All mutations were confirmed by sequencing from the opposite direction.

EXAMPLE IV
ESTIMATION OF MUTATION LOAD AND
DETERMINATION OF ALLELE DROPOUT AND POLYMERASE ERROR

[0043] Using the technique detailed in Example II, a 2 kb segment in the human p53 gene spanning exons 5 to 9 was amplified with 60 to 80% success rate. Mutations were identified in 50% of amplified samples with p53 and/or PCNA positive staining, and are listed in Table 3. Figure 3 shows examples of identified somatic mutations. Panels A through D are examples of identified somatic mutations in sequence analysis. Mutations of C-T, G-A, T-A and T-C were identified with second peak heights from 100% to 30% of the wild type. Causative mutations were found in 50%-70% of the amplified single cells.

Table 3. List of Identified mutations^a

Mutation No.	P53/PCNA Stain	Mutation Type	Wt Base	Mt Base	Base Position	AA Wt	AA Mt	Codon	Conservation
1	+/-	missense	A	G	13074	Lys	Arg	132	Y
2	+/-	missense	G	A	13139	Gly	Ser	154	Y
3	+/-	missense	A	G	14015	Thr	Ala	230	Y
4	+/-	missense	G	C	14061	Gly	Arg	245	Y
5	+/+	missense	C	T	13125	Ser	Phe	149	N
6	+/-	missense	G	T	13409	Val	Leu	217	Y
7	+/-	missense	C	A	14568	Pro	His	300	N
8	+/-	missense	G	A	13229	Asp	Asn	184	N
9	+/-	missense	T	G	14743	Phe	Ser	328	Y
10	+/-	missense	G	A	14574	Gly	Glu	302	Y
11	+/-	missense	A	G	13086	Glu	Arg	136	N

12	+/-	missense	G	T	14699	Ser	Ile	313	N
13	+/-	missense	T	G	14743	Phe	Ser	328	Y
14	+/-	Insertion		A	13607	IVS 6 + 174			
15	+/-	missense	G	A	14574	Gly	Glu	302	N

[0044] a. All of the above were identified from 15 normal nontumorous colon cells with *p53* and PCNA double staining. The missense mutations are either at a conservative site or are found in the *p53* mutation database (<http://www.iarc.fr/P53/index.html>). Mutations were also identified in other normal cells of breast, lung, kidney and gallbladder.

[0045] Each allele has two single stranded templates in the heterozygous *p53* genomic DNA in diploid cell. "Allele dropout" is defined herein as the situation in which the peak of one allele was less than 10% of the second peak at the heterozygous base position. The size effect of single stranded templates on allele dropout is summarized in Table 2 above. As shown, a low level of allele dropout due to nicked template can be achieved by maintaining 6 kb or more of single stranded template.

[0046] False positive mutations may result from a polymerase error in any of the first several PCR cycles. A false positive mutation is identified when a second peak is higher than 10% of the wild type peak in a wild type sample. The two enzyme system with Taq and GB-D DNA polymerases is estimated to have an error rate of 8.5×10^{-6} substitutions per base, this is expected to cause false positive mutation calls in 7% to 12% of the amplified samples or at 4×10^{-5} to 7×10^{-5} chance per nucleotide when amplified from four to one single stranded template.